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Performance of the AOAC Use-Dilution Method with Targeted Modifications: Collaborative Study

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The U.S. Environmental Protection Agency (EPA), in collaboration with an industry work group, spearheaded a collaborative study designed to further enhance the AOAC use-dilution method (UDM). Based on feedback from laboratories that routinely conduct the UDM, improvements to the test culture preparation steps were prioritized. A set of modifications, largely based on culturing the test microbes on agar as specified in the AOAC hard surface carrier test method, were evaluated in a five-laboratory trial. The modifications targeted the preparation of the *Pseudomonas aeruginosa* test culture due to the difficulty in separating the pellicle from the broth in the current UDM. The proposed modifications (i.e., the modified UDM) were compared to the current UDM methodology for *P. aeruginosa* and *Staphylococcus aureus*. *Salmonella choleraesuis* was not included in the study. The goal was to determine if the modifications reduced method variability. Three efficacy response variables were statistically analyzed: the number of positive carriers, the log reduction, and the pass/fail outcome. The scope of the collaborative study was limited to testing one liquid disinfectant (an EPA-registered quaternary ammonium product) at two levels of presumed product efficacies, high and low. Test conditions included use of 400 ppm hard water as the product diluent and a 5% organic soil load (horse serum) added to the inoculum. Unfortunately, the study failed to support the adoption of the major modification (use of an agar-based approach to grow the test cultures) based on an analysis of method's variability. The repeatability and reproducibility standard deviations for the modified method were equal to or greater than those for the current method across the various test variables. However, the authors propose retaining the frozen stock preparation step of the modified method, and based on the statistical equivalency of the control log densities, support its adoption as a procedural change to the current UDM. The current UDM displayed acceptable responsiveness to changes in product efficacy; acceptable repeatability across multiple tests in each laboratory for the control counts and log reductions; and acceptable reproducibility across multiple laboratories for the control log density values and log reductions. Although the data do not support the adoption of all modifications, the UDM collaborative study data are valuable for assessing sources of method variability and a reassessment of the performance standard for the UDM.

The U. S. Environmental Protection Agency (EPA) regulations specify that efficacy data must be submitted to support the registration of antimicrobial products bearing claims to control microorganisms on inanimate surfaces that pose a threat to human health. The registrant must ensure that the product is tested in accordance with the label directions. The AOAC use-dilution methods (UDMs; 1–3), **955.14** (*Salmonella enterica*), **955.15** (*Staphylococcus aureus*) and **964.02** (*Pseudomonas aeruginosa*), are standard methods used to substantiate efficacy claims for liquid formulations. With the exception of the test microbe and the preparation of test culture, the three UDMs are essentially the same. Furthermore, to monitor product performance following registration, the EPA uses the UDMs where applicable in its post registration product stewardship program.

Historically, UDM has been the subject of criticism due to vagueness of the published standards and technique-sensitive aspects of the method, which have been cited as likely sources of variability in the efficacy results. Two significant multilaboratory studies by Cole and Rutala (4) and Cole et al. (5) demonstrated the occurrence of variability among laboratories and identified major aspects of the method deemed as likely sources of the variability, most notably irregular surfaces of the stainless steel carriers (penicylinders), inconsistent inoculum levels, and inadequate removal of the pellicle associated with *P. aeruginosa* test cultures.

Standardization and monitoring of carrier loading were not addressed in those studies; this aspect may have contributed to variability in the efficacy results. Furthermore, the misinterpretation and the subjective nature of the UDM probably have contributed to a wide range of working protocols, i.e.,

standard operating procedures (SOPs), making the comparison of multilaboratory data even more challenging.

An initiative designed to resolve concerns with the UDM led to the development and validation of AOAC *Official Method*SM **991.47**, the hard surface carrier test (HSCT; 6, 7), which is also accepted by EPA to support the registration of liquid disinfectants. However, the HSCT has been used sparingly by the regulated community, purportedly due to the lack of hard water and organic soil load in the validation study.

Over the past several years, the EPA has led an effort to improve the UDM by using the official AOAC INTERNATIONAL method revision process. As a result, a revised version of the UDM was published in the AOAC *Official Methods of Analysis* in 2006. The editorial revisions focused on updating sources of reagents and supplies and increasing specificity of the procedures by providing concise, easy to follow steps without extensive cross-referencing. Recently, significant improvements to the UDM were made by introduction of a procedure to assess the number of viable cells per dried inoculated control carriers and a minimum carrier density (geometric mean density of 10^6 across 6 control carriers) requirement for *S. aureus* and *P. aeruginosa* (8). The revisions were approved by AOAC in 2009 and are presently First Action status revisions. Recently, a procedural modification was approved by AOAC to establish a maximum geometric mean log density of 10^7 per carrier for methods **955.15** and **964.02** (9). As a result of the control carrier count revisions, the UDM may be characterized as a semiquantitative procedure, rather than a strict qualitative one, thus allowing for additional statistical analyses and interpretation of results. In the current report, in addition to analyzing the number of positive carriers directly, the availability of the control counts allows the log reduction for each UDM test to be estimated directly, thereby determining the variability of the UDM with respect to log reductions. This calculation allows for a direct comparison of the variability observed for the UDM to quantitative tests published in the literature (10). Although this approach may be considered new for the analysis of semiquantitative efficacy data, it has been implemented in previous studies of the UDM (11) and the HSCT (12).

EPA is pursuing the adoption of a quantitative efficacy test method based on American Society for Testing and Materials method 2197-02 (13) to replace the UDM for measuring the performance of antimicrobial products. The methodology under consideration is the result of an international harmonization initiative under the purview of the Organization of Economic and Cooperative Development (OECD). Implementation of the new methodology, the OECD Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard Non-Porous Surfaces, and the development of quantitative performance standards may require several years to complete. Thus, as an interim measure, efforts to enhance the performance of the UDM by introducing targeted procedural changes are justified and should be undertaken.

In collaboration with an industry-based technical work group, EPA submitted a survey to a wide range of laboratories that conduct the UDM to help identify and prioritize areas of different practices and interpretation of the procedure. Based on the feedback, methods for the preparation and harvest of the test cultures were determined to be the most divergent among laboratories. Thus, modifications associated with the preparation and harvest of the test cultures were prioritized for this study.

Most importantly, the modifications targeted the preparation of the *P. aeruginosa* test culture due to the difficulty in separating the pellicle from the broth. The potential effects of the pellicle have been documented previously (14, 15). Although the UDM method **964.02** provides specific instructions for avoiding the disruption of the pellicle during the harvesting process, concerns were raised over the potential impact of microscopic pellicle fragments associated with final test cultures on the variability of product performance data. To provide the data necessary to support procedural changes and to assess improvement in method performance (i.e., reduction in method variability), a collaborative study was conducted to compare the results of the current version of the UDM to a modified version. The proposed modifications were classified as procedural changes by AOAC, thus requiring a multilaboratory evaluation. The targeted modifications were largely based on the HSCT methodology.

For this study, *S. aureus* and *P. aeruginosa* were prioritized due to their regulatory importance associated with hospital disinfectant claims. EPA's SOP for conducting the UDM, SOP MB-05 (16), is based on a strict interpretation of the published UDM, and was followed by the participating labs. The modified version of the UDM was the current method with two modifications: (1) use of frozen stock cultures (stored at -70°C or below) instead of stock cultures grown and stored on agar slants and stabs; and (2) the use of an agar-based procedure to grow the test microbes as a means to mitigate the impact of pellicle. The modified UDM included both modifications; thus, the modifications were not evaluated separately.

This paper reports the results of this collaborative study. This report includes comparisons of the two UDM methods with respect to the control counts of viable organisms on the inoculated carriers, the number of positive carriers out of 60, and the log reductions. For each UDM method, the repeatability across tests in each laboratory and the reproducibility across laboratories are compared to results from other published disinfectant methods.

Collaborative Study

Applicability

The scope of this study was limited to testing a liquid disinfectant against *S. aureus* and *P. aeruginosa* on a hard, nonporous surface (stainless steel penicylinders). The generation of the product performance data in this study for an EPA-registered product was for research purposes only. Therefore, the product used in the study was an experimental component only and was not evaluated to support or verify product label claims. The test conditions included the use of 400 ppm hard water as the product diluent and a 5% organic soil load (horse serum) added to the inoculum.

Collaborators and Quality Management

Five volunteer laboratories (denoted as A, B, C, D, and E) participated in this study: one federal laboratory; one contract laboratory; and three industry laboratories. The laboratories had existing microbiology programs, appropriately trained personnel, and had the capability of conducting the protocol within the established timeframe. The EPA Biological and Economic Analysis Division-Microbiology Laboratory Branch,

Fort Meade, MD, facilitated the collaborative study and served as the lead laboratory. The participating laboratories had a history of conducting the UDM and were assumed to be representative of laboratories that routinely conduct the UDM. Analysts performing the tests were asked to familiarize themselves with each version of the UDM method and to build proficiency by the use of in-house practice sessions. Following data collection, the data sheets were submitted in a blind-coded fashion to an industry liaison for initial peer review, and were then forwarded to the EPA study director, who performed a final peer review and submitted the data in spreadsheet format to the statisticians for statistical analysis. Thorough documentation was maintained to ensure that the study was supported by complete, accurate, and chronological records from initial collection of raw data to final analysis and reporting of results. The level of quality assurance was consistent with data collection principles specified under EPA Good Laboratory Practice Standards – FIFRA 40 CFR Part 160 (17).

Study Design

The study design was consistent with past AOAC-approved method modification and validation studies conducted by EPA; however, AOAC did not officially facilitate the process. *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 15442) were the test microbes. EPA SOP MB-05, based on the 2009 revised AOAC *Official Methods* **955.15** and **964.02**, was considered the current UDM. The current UDM and the modified UDM method (which included the two proposed modifications) were conducted side-by-side on each test day so that the results could be compared directly.

One representative EPA-registered quaternary ammonium-based product was used as the experimental test chemical. The product's label ingredients were: (1) 6.510% octyl decyl dimethyl ammonium chloride; (2) 3.255% dioctyl dimethyl ammonium chloride; (3) 3.255% didecyl dimethyl ammonium chloride; and (4) 8.860% alkyl (50% C₁₄, 40% C₁₂, and 10% C₁₆) dimethyl benzyl ammonium chlorides. Screening studies were conducted prior to the official study to determine the product test conditions. The goal was to establish a range of product efficacy to measure the responsiveness of the methods, an important method performance criterion. On each test day, a presumed high-efficacy level of the product was tested using both versions of the UDM, followed by testing of a presumed low-efficacy level of the product by both methods. Product dilution for the high-efficacy level was based on the product's label claim (1:256) and was the same for both organisms; however, for the low-efficacy level, dilutions of 1:576 and 1:1280 were used for *P. aeruginosa* and *S. aureus*, respectively. The higher product dilution for *S. aureus* was necessary due to the microbe's greater sensitivity to the product. The product label claims were used to determine the test conditions for the organic burden, diluent, contact time and temperature. The diluent was sterile 400 ppm (as calcium carbonate) AOAC synthetic hard water prepared per AOAC *Official Method* **960.09** (18). Organic burden in the form of 5% (v/v) horse serum was added to the inoculum prior to carrier inoculation.

The product was tested with a 10 min contact time at a temperature of 20 ± 1°C. The neutralizer for the product was confirmed prior to use and consisted of letheen broth with 2× lecithin plus polysorbate 80. A single tube of

neutralizer/subculture medium was used per carrier. Physically and biologically screened carriers were utilized in the study. The product was distributed to the testing laboratories in containers that were marked only with a treatment code. A single lot of the test chemical was used. In advance of testing, an analysis of formulation chemistry was performed to confirm the level of active ingredients. EPA chemists (Biological and Economic Analysis Division Analytical Chemistry Branch) were responsible for the formulation chemistry, sample labeling, and shipment.

The laboratories were instructed to perform the same number of tests/day: four 60-carrier tests. The set of four 60-carrier tests was replicated on three separate test days/microbe to provide data for an assessment of within-laboratory variability. The order of testing against *S. aureus* and *P. aeruginosa* was randomized for each laboratory; however, the high-efficacy treatment was always performed first on each test day to mitigate cross-contamination to the low-efficacy test carriers. Therefore, each of the five laboratories conducted a total of 24 60-carrier UDM tests. The study director allowed an alternative approach in which a laboratory could conduct two 60 carrier tests/day, consisting of the current and modified methods for each high and low treatment. Under this scenario, an extra 24 h test microbe subculture was necessary to extend the use period of the test culture. Three untreated control carriers for each method were assayed each test day to measure the density of viable bacteria/carrier [expressed as the geometric mean colony forming units (CFUs)/carrier]. The evaluation of the control carriers was performed according to EPA SOP MB-05. An arithmetic mean minimum level of 6 log₁₀ (CFU/carrier), which corresponds to a geometric mean CFU/carrier minimum of 1 × 10⁶, was expected for both test microbes. Testing was repeated if the minimum control carrier counts fell below the 6 log₁₀(CFU/carrier) minimum.

UDM Modifications Evaluated in the Collaborative Study

A single lyophilized ampule of each microbe was used by each laboratory to initiate two types of stocks (one for the current UDM and one for the modified UDM) on the same day. Briefly, for the current UDM, stocks of *S. aureus* were established and stored on nutrient agar slants; *P. aeruginosa* stocks were established and stored on cystine trypticase agar. Final test cultures for the current UDM were prepared in nutrient broth with a minimum of three consecutive 24 h transfers (10 mL for daily transfers) followed by a final 48–54 h 20 mL test culture. For the modified UDM, frozen stocks and final test cultures were prepared as described below.

Preparation of frozen stock cultures for the modified procedure.—Using a tube containing 5–6 mL of tryptic soy broth (TSB), aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix well. Incubate for 24 ± 2 h at 36 ± 1°C. Using a sterile spreader, inoculate a sufficient number of tryptic soy agar (TSA) plates (e.g., 5 to 10 plates/organism) with 100 µL each of the culture. Incubate plates at 36 ± 1°C for 24 ± 2 h. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol) to the surface of each agar plate. Resuspend the cells in this solution using a sterile spreader or sterile swab, and aspirate

the cell suspension from the surface of the agar. Transfer suspension into a sterile vessel. Repeat by adding another 5 mL of cryoprotectant to the agar plates, resuspend the cells, aspirate suspension, and pool with the initial cell suspension. Alternately, 10 mL of cryoprotectant solution may be added/plate for re-suspending with subsequent aspiration. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, pipette approximately 1.0 mL of the diluted suspension into cryovials (e.g., 1.5 mL). Place and store cryovials in -70°C or below freezer.

Final test culture preparation for the modified procedure.—Test culture preparation was consistent with AOAC *Official Method* 991.47. To prepare the final test cultures, defrost a single cryovial at room temperature and briefly vortex. Add 100 μL of the thawed frozen stock to one 20×150 mm tube containing 10 mL nutrient broth and incubate at $36 \pm 1^{\circ}\text{C}$ for 24 ± 2 h. Using a sterile pipet, add 0.1 mL of 24 ± 2 h culture to each of five TSA plates and spread culture onto plate to make a lawn. Incubate 24 ± 2 h at $36 \pm 1^{\circ}\text{C}$. To each plate add 10 mL nutrient broth. With sterile spreader or swab, gently swab surface of plate to remove growth. With sterile pipet, collect nutrient broth containing suspended bacterial cells, and pool in sterile glass flask. Pass suspension, under vacuum, through 47 mm sterile absorbent pads using sterile filtration unit. Use sterile absorbent pads with dispenser (47 mm diameter, cellulose fiber pads; Millipore Corp., Billerica, MA). Collect the suspension in a sterile flask. Sonicate (ultrasonic cleaner) 5 min and let stand for 10 min. Water level in sonicator must be at same level as suspension in flask. Flask must contact bottom of sonicator. Use a timer for tracking time. Use the suspension as soon as possible and only on the day it is prepared. Refrigerate suspension when not in use. Using nutrient broth as the diluent, adjust the test suspension using optical density (OD) reading at 650 nm to a level comparable to the OD (± 0.05) recorded for the final test culture for the current UDM. Use nutrient broth as a blank for assessing the OD. The adjusted suspension represents the final test culture for the modified method. Proceed with inoculation of carriers as indicated in the current UDM.

Statistical Analysis

Data

The data were peer-reviewed by an industry liaison and EPA and submitted for analysis as Excel files for each laboratory. The worksheets were merged into two combined data matrixes, one for the control carrier data and one for the efficacy data. The two data matrixes are displayed in Appendices 1 and 2.

The density of bacteria on each control carrier was recorded as CFU/carrier by the laboratory technician, and then $\log_{10}$ -transformed to form the carrier log density [$\text{LD} = \log_{10}(\text{CFU/carrier})$]. The term *TestLD* is used to denote the mean log density across the three carriers in a single test.

Three efficacy response variables were analyzed: the number of positive carriers, the log reduction, and the pass/fail outcome. The traditional efficacy response for the UDM is the number of positive carriers (denoted by *NP*) among the 60 treated carriers in a test. When interpreting this efficacy response variable, it is important to remember that a high number of positive carriers indicates low disinfectant efficacy.

Comparisons based only on the number of positive carriers

are somewhat limited and flawed because they do not take into account the viable cell counts associated with the inoculated carriers. The log reduction (LR), the usual measure of efficacy for quantitative disinfectant tests, accounts for any differences in the number of viable cells inoculated on the carriers in different tests. Consequently, being able to measure the efficacy of the UDM in terms of LRs enables one to compare the variability of the UDM to literature values for other quantitative or semiquantitative disinfectant tests. This was the approach taken in an analysis of the UDM by Tomasino and Hamilton (11), and for the HSCT by Hamilton et al. (12). The LR for a single test is calculated by subtracting the mean log density for treated carriers from the mean log density for control carriers (19). For the UDM method, viable cell counts on the treated carriers are not available for directly calculating the mean log density for the treated carriers, but that mean can be estimated from the number of positive carriers out of 60, which yields the following equation for the LR based on a modified most probable number formula (20),

$$\text{LR} = \text{TestLD} - \log_{10}[-\ln((60.5 - \text{NP})/61)].$$

The pass/fail outcome is based on the performance standard for which a pass is recorded if there are 0 or 1 positive carriers out of 60 tested.

For each of the response variables (control LDs, number of positive carriers, LRs, pass/fail outcomes) considered in the collaborative study, diagnostic plots were used to detect the occurrence of outliers and inconsistent or unusual values. All such data were brought to the attention of the study director and subsequently validated; no data were rejected. Two data-related issues were noted: (1) in a few cases, a laboratory repeated a test because the control carrier counts were too low; and (2) the number of control carriers observed on each test day was sometimes three and sometimes six, but always the same number for both methods. The first issue arose because the study protocol specified that the geometric mean density (CFU/carrier) must be at least 1×10^6 . When the geometric mean density was below 1×10^6 , the test was repeated. If a laboratory repeated a test for one method, it also repeated the test for the other method, even if the geometric mean density for the other method was at least 1×10^6 . In one instance, a test was repeated a second time. For these reasons, the initial data set contained multiple results for some combinations of microbe, efficacy level, replicate, and laboratory. After consultation with the study director, it was determined that if a test was repeated, only the data from the final repeat would be analyzed. Otherwise, all submitted data were included in the analysis. The second issue did not require a decision, but it created an unbalanced data set because some test days produced one set of three inoculated, untreated carrier counts, and other test days produced two sets of three carrier counts. This issue required that the statisticians fit the analysis of variance (ANOVA) models (described in the next section) using the method of restricted maximum likelihood (REML), which is suitable for unbalanced data (21).

Method Performance Attributes (Resemblance, Responsiveness, Repeatability, and Reproducibility)

The current and modified methods were evaluated separately to assess the extent to which each method achieved the desirable

attributes of resemblance, responsiveness, repeatability, and reproducibility. Each of these four attributes is defined next.

The inoculated carriers used in different tests and in different laboratories should resemble each other; that is, that the bacteria-laden carriers for the UDM tests should be similar from test to test. Resemblance was assessed by calculating standard deviations (SDs) for the control carrier log densities, both within and across laboratories. Small SDs indicate good resemblance of the untreated control carriers. The SDs were calculated by an ANOVA, which estimated four nested variance components: the variance among carriers within a test (CS^2); the variance among replicate tests within the same test day ($CS_{Rep(Day)}^2$); the variance among multiple test days in a laboratory (CS_{Day}^2); and the variance among laboratories (CS_{Lab}^2). The resemblance repeatability SD

$$CS_r = \sqrt{CS^2/3 + CS_{Rep(Day)}^2 + CS_{Day}^2}$$

is a measure of the within-laboratory variability of the *TestLD* values. The resemblance reproducibility SD

$$CS_R = \sqrt{CS_r^2 + CS_{Lab}^2}$$

is a measure of the total variability of the *TestLD* values across all laboratories. Some guidance on the acceptable range of values for the repeatability SD (CS_r) and the reproducibility SD (CS_R) for standardized disinfectant tests is provided by a review of historical data (22, 23). A value of $CS_r < 0.5$ was found to be acceptable; CS_R values < 0.7 were found to be acceptable.

A method should be responsive to changes in disinfectant efficacy. In this collaborative study, two presumably different efficacy levels (low- and high-efficacy) of the disinfectant were tested by both UDM methods on the same test day. That parallel testing allowed direct comparison of the test results for the low- and high-efficacy levels. One way that responsiveness was measured was by the difference in the percentage of positive carriers between the two efficacy levels on each test day

$$RespNP = P_L - P_H$$

where the low-efficacy percentage of positive carriers out of 60 is denoted by P_L and the high efficacy percentage of positive carriers out of 60 is P_H . Thus, the low- and high-efficacy levels performed on the same day for the same microbe and method were paired. If *RespNP* is significantly larger than zero, then we conclude that the method is responsive. For each combination of method and microbe, a one-sided 95% lower confidence limit for the true mean responsiveness was calculated, along with the *P*-value for a one-sided test of the true responsiveness. The calculations are based on modified Price-Bonett binomial techniques (24) for linear combinations of binomial responses, which uses a normal approximation to the binomial distribution, an approximation that is justified for this study because each proportion is calculated from 60 carriers.

Responsiveness was also measured by the difference in LR values between the two efficacy levels on each test day

$$RespLR = LR_H - LR_L$$

where the low-efficacy LR is denoted by LR_L , and the high-efficacy LR is LR_H . For each combination of microbe and method, an ANOVA was fitted to the *RespLR* values, with laboratory as a random effect. Based on the variance components estimated

Table 1. Statistical summary for control *TestLD* values for the current and modified use-dilution methods

Microbe	Method	Mean (SEM) ^a	CS_r	CS_R
<i>P. aeruginosa</i>	Current	6.71 (0.14)	0.25	0.38
<i>P. aeruginosa</i>	Modified	6.68 (0.09)	0.36	0.37
<i>S. aureus</i>	Current	6.64 (0.12)	0.16	0.30
<i>S. aureus</i>	Modified	6.73 (0.09)	0.14	0.24

^a Averaged across laboratories, replicates, and tests for each combination of microbe and method.

from this ANOVA, a lower 95% confidence limit was calculated using a *t*-distribution with four degrees of freedom to ascertain whether the mean *RespLR* was statistically significantly greater than zero.

A disinfectant test method is deemed repeatable and reproducible if independent tests of the same treatment produce a similar efficacy response. Repeatability pertains to independent tests in a single laboratory. Reproducibility pertains to independent tests in different laboratories. In this study, LR was the only efficacy response used to assess repeatability and reproducibility since the LR response is directly comparable to results reported in the literature. The LR values for each combination of method, microbe, and efficacy level, were analyzed by a one-way random effects ANOVA to estimate the repeatability (or within-laboratory) variance S_r^2 and the among-laboratory variance S_{Lab}^2 (23). The repeatability SD S_r includes within-test variability and among-test variability for tests conducted in the same laboratory. The reproducibility SD, $S_R = (S_r^2 + S_{Lab}^2)^{1/2}$, includes among-laboratory variability, as well as the variability that makes up S_r . Note that SD is a measure of imprecision; therefore, small values of S_r and S_R , typically calculated from collaborative studies of quantitative disinfectant tests, indicate good repeatability and reproducibility, respectively.

Some guidance on the acceptable range of S_r and S_R for standardized disinfectant tests is provided by a literature review (9). The review found that the LR values from accepted, standardized suspension and dried surface tests had S_R that ranged from 0.3 to 1.5, with a median of 0.9, and S_r that ranged from 0.2 to 1.2, with a median of 0.5. Typically, 50% of the total variance of LR values (S_R^2) was attributable to the variance among laboratories. More recently, Tomasino et al. (8) reported S_r that ranged from 0.22 to 0.24 and S_R that ranged from 0.29 to 0.32 for the UDM. These observations can be used as rough guidelines for evaluating the UDM test methods presented in this report.

Throughout the analyses, periodic checks were conducted

Table 2. The mean difference and 90% confidence interval for the true mean difference between concurrently observed *TestLD* values (current–modified), presented for each microbe

Microbe	Mean <i>TestLD</i>		<i>TestLD</i> difference: current–modified		
	Current	Modified	Mean	Lower limit	Upper limit
<i>P. aeruginosa</i>	6.71	6.68	0.03	−0.25	0.31
<i>S. aureus</i>	6.64	6.73	−0.08	−0.36	0.19

Figure 1. Number of positive carriers observed for *P. aeruginosa*. Each point is the result from a single test. The horizontal lines indicate the mean number of positive carriers for the low- and high-eff cacy levels for each of the two methods; the mean value is presented above the line.

to ensure that the data were not inadvertently altered during statistical manipulations. Normal probability and residual plots were used to determine that LDs and LR_s satisfied the normality and homogeneity of variance assumptions required by the ANOVA models. The ANOVA models were fit by REML using the *nlme* package in the software R (v. 2.11.0). All statements of statistical significance are made with respect to a significance level of 5%.

Method Comparison (Equivalence Testing)

An important goal of the statistical analysis was to assess the equivalence of the two methods with respect to the following responses: the LD_s of the untreated controls; the number of positive carriers; and the LR_s. The assessment was based on statistical equivalence tests which determine if the observed differences between the two methods were negligibly small (11). In the equivalence tests, the following hypotheses were compared:

$$H_0: |\mu_{\text{current}} - \mu_{\text{modified}}| \geq \delta$$

$$H_a: |\mu_{\text{current}} - \mu_{\text{modified}}| < \delta$$

The parameter μ_{modified} is one of the true mean responses estimated in this study for the modified UDM (i.e., either the true mean control log density, the true percentage of positive carriers, or the true mean LR), and the parameter μ_{current} is the corresponding true mean UDM response for the current method. The equivalence parameter δ specifies the largest difference in

Figure 2. Number of positive carriers observed for *S. aureus*. Each point is the result from a single test. The horizontal lines indicate the mean number of positive carriers for the low- and high-eff cacy levels for each of the two methods; the mean value is presented above the line.

the responses that is negligible and not of practical importance. The null hypothesis H_0 is rejected in favor of the hypothesis H_a for equivalence of the two methods at a 5% significance level when the 2-sided 90% confidence interval for $\mu_{\text{current}} - \mu_{\text{modified}}$ is contained in $(-\delta, \delta)$ (25).

When comparing the true mean control log densities or the true mean LR_s, the difference between the two methods was calculated for each test day; an ANOVA was then fit to these differences with laboratory as a random effect. Based on the variance components estimated from this ANOVA, a 90% confidence interval for the true mean difference was calculated using a *t*-distribution with four degrees of freedom. When comparing the true percentage of positive carriers, the difference in percentages between the two methods was calculated for each test day; the Price-Bonett technique (24) was then applied to construct a 90% confidence interval for the true difference.

For this UDM collaborative study, equivalence tests of the mean log densities of the untreated controls were conducted with $\delta = 0.40$. Thus, absolute mean differences in the log densities of the viable organisms on the inoculated carriers as large as 0.40 on the $\log_{10}(\text{CFU}/\text{carrier})$ scale (which corresponds to a multiple of 2.5 on the CFU/carrier scale) were considered negligible and not of practical importance. Equivalence tests of the percentage of positive carriers were conducted with $\delta = 5\%$. Thus, absolute differences as large as 5% (i.e., differences up to three carriers) were considered negligible and not of practical importance. Equivalence tests of the LR_s were conducted with $\delta = 0.5$. Thus, absolute mean differences in the LR_s as large as 0.5 were considered negligible and not of practical importance.

Table 3. Comparison of efficacy based on the number of positive carriers

		Percentage positive				
		Current method	Modified method	Comparison of efficacy: $P_{Cur}-P_{Mod}$		
Microbe	Efficacy level	P_{Cur} , %	P_{Mod} , %	Mean, %	90% 2-sided CI lower limit, % ^a	90% 2-sided CI upper limit, % ^a
<i>P. aeruginosa</i>	High	2.2	5.1	-2.9	-4.3	-1.5
<i>P. aeruginosa</i>	Low	31.3	43.9	-12.6	-15.7	-9.4
<i>S. aureus</i>	High	1.6	1.4	0.2	-0.8	1.1
<i>S. aureus</i>	Low	9.3	6.6	2.8	0.7	4.8

^a Calculated using the Price-Bonett technique.

Figure 3. Assessment of method responsiveness for *P. aeruginosa* where each point represents the number of positive carriers from a single test/method. The lines connect the low- and high-eff cacy levels for each method.

Results and Discussion

Comparison of the Untreated Control Carrier Counts (*TestLD* Values)

The control *TestLD* values reported in this study and the repeatability control CS_r and reproducibility control CS_R values were very similar to those previously reported (8). The mean control *TestLD* values, variance components, and CS_r and CS_R SDs for each combination of microbe and method are displayed in Table 1. Two of the *TestLD* values for the modified method marginally fell below 6.0 and were repeated and not included in the analysis; otherwise, all other control *TestLD*s were above the prescribed minimum of 6.0. The mean LDs are almost identical for each combination of microbe and method ranging from 6.64 to 6.73. The use of OD readings to adjust the final test culture preparations for the modified UDM was a valuable tool to ensure that the carrier counts were comparable between the two methods. Thus, the similar *TestLD*s strengthened our ability to compare the performance of the two methods.

Based on the CS_r and CS_R SDs, the control *TestLD*s for *P. aeruginosa* were slightly more variable than those for *S. aureus*; however, both methods exhibited acceptably small S_r (range of 0.14 to 0.36) and S_R (range of 0.24 to 0.38). The statistical analysis of the differences between control *TestLD* values for the two methods, current minus modified, for each pair of control carrier counts is presented in Table 2. The table shows that the confidence limit farthest from zero is -0.36 . Thus, as long as mean $\log_{10}$ (CFU/carrier) differences as large as 0.40 are considered negligible, then the *TestLD* values for the current and modified methods are statistically significantly equivalent. Therefore, although different procedures were used for the

Figure 4. Assessment of method responsiveness for *S. aureus* where each point represents the number of positive carriers from a single test/method. The lines connect the means at low- and high-eff cacy levels for each method.

preparation of the final test cultures, both methods produced acceptable and statistically equivalent control *TestLD*s.

Comparison of Product Efficacy Results Based on the Number of Positive Carriers

For each method, the numbers of positive carriers/test for each laboratory are plotted in Figure 1 for *P. aeruginosa* and in Figure 2 for *S. aureus*. Across efficacy levels and methods, the number of positive carriers observed for *S. aureus* was lower than those for *P. aeruginosa*. Across microbes and methods, the highest degree of variability was noted at the low-eff cacy level. Laboratory D generated a low number of positive carriers for the modified method at the low-eff cacy treatment for *P. aeruginosa*; however, based on a discussion with the laboratory staff and an inspection of the raw data, the data from laboratory D were considered valid and used in the analysis. The number of positive carriers across both methods was far less variable for the high-eff cacy treatment. The product was more dilute for the low eff cacy treatment for *S. aureus* (1:1280) compared to that for *P. aeruginosa* (1:576); thus, our ability to compare the differences in product efficacy between microbes is limited at the low-eff cacy level.

For *P. aeruginosa*, the high eff cacy treatment yielded a similar number of positives (mean of 1.3 positives out of 60 for the current method and 3.1 positives out of 60 for the modified method). However, the overall variability in the results was greater for the modified method: range of 0 to 5 positives for the current method, compared to 0 to 11 for the modified method. Both methods produced a mean of 0.9 positives out of

Table 4. Responsiveness based on the number of positive carriers

Microbe	Method	Percentage positive		Responsiveness: $RespNP = P_L - P_H$		
		Low-eff cacy	High-eff cacy	Mean	95% one-sided CI (lower limit) ^a	P-value, one-tailed ^a
		P_L	P_H			
<i>P. aeruginosa</i>	Current	31.3%	2.2%	29.1%	26.6%	<0.001
<i>P. aeruginosa</i>	Modified	43.9%	5.1%	38.8%	36.3%	<0.001
<i>S. aureus</i>	Current	9.3%	1.6%	7.8%	6.1%	<0.001
<i>S. aureus</i>	Modified	6.6%	1.4%	5.1%	3.6%	<0.001

^a Calculated using the Price-Bonett technique.

Table 5. Mean LR averaged across 15 tests^a

Microbe	Eff cacy level	Mean LR		LR Difference: current–modif ed		
		Current	Modif ed	Mean	90% Conf dence interval	
					Lower limit	Upper limit
<i>P. aeruginosa</i>	Low	7.25	7.11	0.15	−0.60	0.90
<i>P. aeruginosa</i>	High	8.26	8.09	0.17	−0.35	0.70
<i>S. aureus</i>	Low	7.79	8.00	−0.21	−0.48	0.07
<i>S. aureus</i>	High	8.48	8.49	−0.01	−0.25	0.23

^a Three replicates in each of the fve laboratories with the mean difference and the 90% conf dence interval for the true mean difference between LR values (current–modif ed) for each test day, calculated for each combination of microbe and eff cacy level.

60 positives for *S. aureus* for the high treatment with a range of 0–4 positive carriers across the methods.

For *P. aeruginosa*, the mean number of positive carriers for the low-eff cacy treatment was 18.8 and 26.3 for the current and modif ed method, respectively. For the low treatment, the range in positives for *P. aeruginosa* was greater: 9–40 and 0–55 for the current and modif ed methods, respectively. For *S. aureus*, the current method yielded a mean of 5.6 positives for the low eff cacy treatment. Similarly, a mean of 3.9 positives was observed for the low-eff cacy treatment for the modif ed method.

Across all combinations of method, microbe, and eff cacy level, the mean percentages of positive carriers out of 60 for the current method (P_{Cur}), and the modif ed method (P_{Mod}) are presented and compared ($P_{Cur} - P_{Mod}$) in Table 3. The absolute difference in numbers of positive carriers between methods was greatest for the *P. aeruginosa*/low eff cacy combination (12.6% difference, corresponding to 7.6 positives out of 60 carriers). The wide conf dence interval indicates a failure to ascertain statistical equivalence of the two methods for the *P. aeruginosa*/low eff cacy combination. The percentage of positive carriers was very similar across methods for *S. aureus*; an equivalence test shows that the methods produce statistically signif cantly equivalent percentages of positive carriers for both low- and high-eff cacy levels for *S. aureus*, as long as percentages as large as 4.8% (equivalent to 2.9/60 carriers) are considered small and negligible.

These results suggest that the *P. aeruginosa* inoculum generated using the agar-based approach is more tolerant of disinfection than the current broth-based approach and produces more variable results. However, further studies would be necessary to fully characterize the differences. Alternatively, it is possible that the modif ed method as written is more challenging to perform and additional practice runs are necessary to optimize the results. The use of frozen stock cultures is a routine practice used by microbiology laboratories

that conduct standardized test methods, and should not have inf uenced the outcome of the modif ed approach. However, the harvesting and standardization of inoculum using the agar-based approach involves several steps and may be responsible for the increase in variability.

Assessment of Method Responsiveness Based on the Number of Positive Carriers

To assess method responsiveness, the numbers of positive carriers for each microbe and method were plotted to compare the low- and high-eff cacy data side-by-side (Figures 3 and 4). The data are further summarized as percentage of positives with the associated statistics in Table 4. With the exception of laboratory D for *P. aeruginosa*, both methods exhibited the ability to detect the presumed eff cacy-response relationship. A statistical analysis showed that the percentages of positive carriers for all combinations of method and microbe are signif cantly lower ($P < 0.001$) for the high-eff cacy treatment (the mean differences ranged from 5.1% to 38.8%) compared to the low-eff cacy treatment. Thus, both versions of the UDM are statistically signif cantly responsive to the two eff cacy levels of the product.

Comparison of Product Eff cacy Based on LR

The mean LR values for each microbe and method combination are provided in Table 5. The LRs reinforce conclusions based on differences in product eff cacy results using the number of positive carriers. Across the four combinations of microbe and eff cacy level, the mean LR values ranged from 7.1 to 8.5. Absolute mean differences in the LR between the current and modif ed methods across microbes and eff cacy levels were small, ranging from 0.01 to 0.21. Since the conf dence intervals were wide for tests with *P. aeruginosa* (0.9 low- eff cacy upper conf dence limit and a 0.7 high-eff cacy upper conf dence limit),

Table 6. Repeatability SD and reproducibility SD of LR for each of the two methods, for each combination of microbe and eff cacy level

Microbe	Efficacy level	S_r		S_R	
		Current	Modified	Current	Modified
<i>P. aeruginosa</i>	Low	0.15	0.42	0.55	1.10
<i>P. aeruginosa</i>	High	0.33	0.50	0.53	0.53
<i>S. aureus</i>	Low	0.26	0.34	0.36	0.40
<i>S. aureus</i>	High	0.32	0.32	0.32	0.39

Figure 5. Comparison of responsiveness using the LR. Each point is the mean LR across 15 tests (three replications in each of five laboratories). The lines connect the low- and high-eff cacy levels for each method: (a) *P. aeruginosa* tests and (b) *S. aureus* tests.

the collaborative study data fail to support equivalency of the LR values for the current and modified methods applied to *P. aeruginosa*. For tests with *S. aureus*, the narrow confidence intervals show that the mean LR values for the current and modified methods are statistically significantly equivalent as long as absolute mean LR differences as large as 0.5 are considered small and negligible.

The S_r and S_R SDs for the LR are provided in Table 6. For each combination of microbe and efficacy level, both methods produced acceptably small S_r and S_R values (10). Overall, S_r and S_R were less for *S. aureus* tests than for the corresponding *P. aeruginosa* tests. Across the combinations of microbe and efficacy level, the S_r values ranged from 0.15 to 0.33 for the current method and from 0.32 to 0.50 for the modified method; the S_R values ranged from 0.32 to 0.55 for the current method and from 0.39 to 1.10 for the modified method. Based on the S_r and S_R values for the LR, the modifications did not reduce method variability.

Comparison of Responsiveness Based on Assessment of LR

To assess and compare overall method responsiveness with respect to the LRs, the mean LR values across the 15 replicate tests per microbe are plotted in Figure 5. The LR data reinforce conclusions reached on responsiveness using the number of positive carriers. For each microbe, the slopes for both methods are very similar. For *S. aureus*, the low-eff cacy level produced uniformly large LR values, thereby causing

smaller responsiveness measures than for the corresponding *P. aeruginosa* tests. Table 7 presents a statistical summary of the responsiveness measure, including the mean and a 95% one-sided confidence interval for the true mean. For each combination of microbe and method, the lower confidence limit is positive and the P -value is very small. Thus, the mean LR at the high-eff cacy level is statistically significantly larger than the mean LR at the low-eff cacy level ($P \leq 0.021$). This confirms that both methods are highly responsive to changes in product eff cacy.

Assessment of the Pass/Fail Outcome for the Product

The current performance standard (which defines a passing outcome) established for the UDM and accepted by the EPA for liquid disinfectants is 0 to 1 positive carriers out of 60 tested. In this study, the high eff cacy treatment conditions were based on the label claim of the product; thus, the high-eff cacy treatment was expected to pass the UDM and was deemed most relevant to this evaluation. The pass/fail outcome for the high-eff cacy treatment is summarized in Table 8 for *S. aureus* and Table 9 for *P. aeruginosa*. For the *S. aureus* and high eff cacy treatment combination, 10/15 (67%) and 12/15 (80%) tests passed for the current and modified methods, respectively. For *P. aeruginosa* and the high-eff cacy treatment combination, 11/15 (73%) tests with the current method passed while only 6/15 (40%) tests passed for the modified counterpart. For the low-eff cacy treatment, only 3/15 and 2/15 tests passed the current and modified method, respectively, for *S. aureus*. For the *P. aeruginosa* and low-eff cacy treatment combination, the current method passed 0/15 times, while 4/15 tests passed the modified method. These data indicate that the modifications did not improve method performance. Thus, the modifications tested in this study, except the use of frozen stock cultures, will not be pursued as official procedural changes.

Conclusions

Overall, the targeted modifications did not improve the performance of the UDM, i.e., the S_r and S_R for the modified method were equal to or greater than the S_r and S_R for the current method across the various test variables. In fact, it appears that the modifications increased the variability in the results. The control *TestLDs* consistently met the $6 \log_{10}$ (CFU/carrier) minimum and were comparable for both methods. The values for CS_r and CS_R SDs for the *TestLDs* were acceptably small (21, 22), and were similar to data previously published (8).

Table 7. The mean responsiveness measure $RespLR = LR_H - LR_L$ for LR values, the 95% lower, one-sided confidence limit for the true mean $RespLR$, and the one-tailed P -value, presented for each combination of microbe and method

Microbe	Method	Responsiveness: $RespLR$				
		Mean LR		95% Confidence interval		
		High-eff cacy	Low-eff cacy	Mean	Lower limit (one-sided)	P -value (one-tailed)
<i>P. aeruginosa</i>	Current	8.26	7.25	1.01	0.80	<0.001
<i>P. aeruginosa</i>	Modified	8.09	7.11	0.98	0.27	0.021
<i>S. aureus</i>	Current	8.48	7.79	0.68	0.42	0.003
<i>S. aureus</i>	Modified	8.49	8.00	0.49	0.31	0.002

Table 8. Number of positive carriers and Pass/Fail outcome for the *S. aureus*/high QAC eff cacy treatment for current and modif ed UDM

Laboratory	Replication	No. positives out of 60 carriers	
		Current method ^a	Modif ed method ^a
A	1	0 (Pass)	0 (Pass)
	2	0 (Pass)	1 (Pass)
	3	0 (Pass)	0 (Pass)
B	1	0 (Pass)	0 (Pass)
	2	0 (Pass)	1 (Pass)
	3	2 (Fail)	1 (Pass)
C	1	0 (Pass)	0 (Pass)
	2	0 (Pass)	1 (Pass)
	3	0 (Pass)	0 (Pass)
D	1	3 (Fail)	2 (Fail)
	2	3 (Fail)	3 (Fail)
	3	3 (Fail)	4 (Fail)
E	1	0 (Pass)	0 (Pass)
	2	0 (Pass)	0 (Pass)
	3	3 (Fail)	0 (Pass)
		10/15 Pass	12/15 Pass

^a Pass/Fail outcome: 0 to 1 positive out of 60 is a Pass; 2 or more positives out of 60 is a Fail.

Table 9. Number of positive carriers and Pass/Fail outcome for the *P. aeruginosa*/high QAC eff cacy treatment for current and modif ed UDM

Laboratory	Replication	No. positives out of 60 carriers	
		Current method ^a	Modif ed method
A	1	0 (Pass)	10 (Fail)
	2	0 (Pass)	11 (Fail)
	3	0 (Pass)	3 (Fail)
B	1	1 (Pass)	1 (Pass)
	2	1 (Pass)	2 (Fail)
	3	1 (Pass)	1 (Pass)
C	1	3 (Fail)	1 (Pass)
	2	2 (Fail)	2 (Fail)
	3	1 (Pass)	0 (Pass)
D	1	1 (Pass)	0 (Pass)
	2	2 (Fail)	2 (Fail)
	3	1 (Pass)	1 (Pass)
E	1	5 (Fail)	4 (Fail)
	2	1 (Pass)	5 (Fail)
	3	1 (Pass)	3 (Fail)
		11/15 Pass	6/15 Pass

^a Pass/Fail outcome: 0 to 1 positive out of 60 is a Pass; 2 and above positives out of 60 is a Fail.

Furthermore, based on differences between the number of positive carriers and the LR values between high- and low-eff cacy treatments, both methods were deemed responsive to presumed changes in eff cacy. The modif ed method with *P. aeruginosa* generated a 12.6% (7.6 carriers) increase in the number of positive carriers for the low-eff cacy treatment compared to the current method, thus not supporting equivalency. The pass/fail data suggest an increased likelihood of failing an effective product when utilizing the modif ed method for *P. aeruginosa*. A greater range in the number of positives for *P. aeruginosa* was observed for the modif ed method (0–11 carriers) compared to the current method (0–5 carriers) for the high-eff cacy treatment. The corresponding LR values for *P. aeruginosa* also show a decrease in the mean LR for the modif ed method. The difference in positive carriers for *S. aureus* across methods was low and was 0.2% (0 carriers out of 60) for the high treatment and 2.8% (1.7 carriers out of 60) for the low treatment. Thus, the modif cations impacted *S. aureus* much less than *P. aeruginosa*.

The current UDM, as performed in this study, displayed acceptable responsiveness to changes in product eff cacy: acceptable repeatability across multiple tests in each laboratory for the control LDs and the LRs; and acceptable reproducibility across multiple laboratories for the control LDs and the LRs. However, using the pass/fail criterion, one might argue that the method's variability remains too high for reliable use as a regulatory method with the current performance standard of 0–1 positive out of 60, especially for *P. aeruginosa*. These conclusions assume that the product tested is formulated to exhibit a predictable outcome (i.e., results for a "gold" standard product) under repeated testing across several laboratories; however, this conclusion cannot be made without further analysis. Based on the data and the associated statistical analysis provided in this report, a reassessment of the performance standard is being pursued by the EPA.

It is clear that conducting the current UDM using the same standardized procedure with trained analysts will provide more consistent results. Although there may be advantages (e.g., standardized inoculum) for the use of the modif ed method to generate f nal test cultures (i.e., reduce potential impact of the *P. aeruginosa* pellicle on method variability), the data reported here do not support its adoption into the UDM. Thus, pursuing the modif cations, with the exception of the frozen stocks, is not recommended. The HSCT approach generates a test suspension that is highly ref ned and may signif cantly change the quality of the inoculum and/or the physiological state of the test bacteria. These changes may result in differences in bacterial attachment and distribution on the carrier surface, and may be responsible for changes in tolerance to disinfectants, which was most evident in the *P. aeruginosa* data. Further experimentation is required to conf rm this theory. However, the authors propose retaining the frozen stock preparation step of the modif ed method, and based on the statistical equivalency of the control log densities, support its adoption as a procedural change to the current UDM. Furthermore, the use of frozen stock cultures is a widespread practice in microbiology laboratories and will reduce the likelihood of genetic drift due to frequent subculturing of the test bacteria. The proposed revision would eliminate the use of stock cultures grown and stored on nutrient agar slants (955.15) and cystine tryptic agar stabs (964.02), and is provided below.

Reagents

Preparation of frozen stock cultures for the modified procedure.—Using a tube containing 5–6 mL of TSB, aseptically withdraw 0.5–1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix well. Incubate for 24 ± 2 h at $36 \pm 1^\circ\text{C}$. Using a sterile spreader, inoculate a sufficient number of TSA plates (e.g., 5–10 plates/organism) with 100 μL each of the culture. Incubate plates at $36 \pm 1^\circ\text{C}$ for 24 ± 2 h. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol) to the surface of each agar plate. Resuspend the cells in this solution using a sterile spreader or a sterile swab, and aspirate the cell suspension from the surface of the agar. Transfer the suspension into a sterile vessel. Repeat by adding another 5 mL cryoprotectant to the agar plates, resuspend the cells, aspirate suspension, and pool with the initial cell suspension.

Alternately, 10 mL cryoprotectant solution may be added/plate for resuspending with subsequent aspiration. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, pipette approximately 1.0 mL of the diluted suspension into cryovials (e.g., 1.5 mL). Place and store cryovials in -70°C or below freezer. Store these frozen stock cultures up to 18 months.

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